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Hypothesis

Cytochrome c_{6A} is a funnel for thiol oxidation in the thylakoid lumen

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Abstract Cytochrome c_{6A} is a dithio-cytochrome recently discovered in land plants and green algae, and believed to be derived from the well-known cytochrome c_6. The function of cytochrome c_{6A} is unclear. We propose that it catalyses the formation of disulphide bridges in thylakoid lumen proteins in a single-step disulphide exchange reaction, with subsequent transfer of the reducing equivalents to plastocyanin. The haem group of cytochrome c_{6A} acts as an electron sink, allowing rapid resolution of a radical intermediate formed during reoxidation of cytochrome c_{6A}. Our model is consistent with previously published data on mutant plants, and the likely evolution of the protein.

Keywords: Plastocyanin; Disulphide exchange; Cytochrome c_{6A}; Disulphide formation; Thylakoid; Photosystem

1. The discovery of cytochrome c_{6A}

Cytochrome c_{6A} is a *c*-type cytochrome of unknown function occurring in plants and green algae [1]. It is homologous to cytochrome c_6, a thylakoid luminal protein found in cyanobacteria and eukaryotic algae that is responsible for photosynthetic electron transfer between the cytochrome b_6f complex and photosystem I [2]. In cyanobacteria and in green algae such as *Chlamydomonas reinhardtii*, this function is carried out when copper is available by the soluble, copper-containing protein plastocyanin [3–5]. It was generally accepted that land plants use only plastocyanin for electron transfer between the cytochrome b_6f complex and photosystem I, and lack cytochrome c_6. However, in 2002, Wastl et al. and Gupta et al. reported independently that a protein with sequence similarity to cytochrome c_6 indeed exists in land plants [6,7]. Wastl et al. subsequently proposed the name cytochrome c_{6A} for the protein and showed [8] that *C. reinhardtii* contains genes for both cytochrome c_{6A} and cytochrome c_6. Although Gupta et al. initially suggested that cytochrome c_{6A} can substitute directly for plastocyanin in photosynthetic electron transfer, experiments using *Arabidopsis* lines with mutations in the plastocyanin genes, as well as studies on electron transfer in vitro have contradicted this [9,10]. The role of cytochrome c_{6A} remains unclear [1,11], but we propose here a function in the formation of disulphide bridges in the thylakoid lumen.

2. What is known about cytochrome c_{6A}

A number of different experimental approaches have provided information about cytochrome c_{6A}, which any satisfactory hypothesis for its function needs to accommodate. We summarize this information here.

Lack of cytochrome c_{6A} has no obvious phenotype in wild-type plants, but is lethal when plastocyanin levels are reduced. Gupta et al. showed that *Arabidopsis* plants with a mutation in the cytochrome c_{6A} gene appeared normal, that plants in which plastocyanin gene expression had been reduced by RNAi showed reduced growth, and that the combination was lethal [7]. Weigel et al. showed that complete loss of plastocyanin was lethal [9], even in the absence of mutations affecting cytochrome c_{6A}. Howe et al. [1] therefore suggested that cytochrome c_{6A} had a signalling role, allowing plants to respond to stress induced by lack of plastocyanin. However, recent structural data indicate that a signalling role is unlikely (Marcaida et al., submitted). We suggest an alternative interpretation here, that the normal function of cytochrome c_{6A} can be fulfilled by plastocyanin as long as the latter is present at normal levels.

Cytochrome c_{6A} interacts with a disulphide-containing immunophilin in the thylakoid lumen. Gupta et al. found cytochrome c_{6A} through its interaction with the luminal immunophilin FKBP13 in a yeast two-hybrid assay [7]. This, together with biochemical fractionation experiments, analysis of predicted targeting sequences, and reaction in vitro with plastocyanin, suggests a location in the lumen.

Cytochrome c_{6A} is present at low levels. The protein has not yet been reported directly, either through spectroscopic analysis of chloroplasts, or, as far as we are aware, in proteomic studies – including those of the thylakoid lumen (reviewed in [1]). Microarray experiments suggest that the tissue distribution of mRNA for cytochrome c_{6A} resembles that for plastocyanin, but at much lower levels [1].

Cytochrome c_{6A} has an insertion containing conserved cysteine residues. Comparison of the predicted amino-acid sequences of cytochrome c_{6A} and cytochrome c_6 from a range of sources indicated that cytochrome c_{6A} has an insertion of twelve amino acids in a loop region (the loop insertion peptide, LIP) compared to cytochrome c_6. There are two absolutely conserved cysteine residues within the LIP [6,8].

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Cytochrome c_{6A} cannot act as an electron carrier from cytochrome f to photosystem I, but can react with plastocyanin. Analysis of cytochrome c_{6A} expressed in *E. coli* indicated that it has a redox midpoint potential too low to allow it to accept electrons from cytochrome *f*, in contrast with plastocyanin and cytochrome c_6 [10]. Site-directed mutagenesis showed this change in potential was not due directly to the cysteine residues in the LIP, or to the LIP as a whole [12]. Although the midpoint potential of cytochrome c_{6A} would be consistent with a reaction with photosystem I, studies in vitro again showed that the direct reaction occurred a hundred times more slowly than the reaction between plastocyanin and photosystem I [10]. Howe et al. suggested [1] that cytochrome c_{6A} might be able to pass electrons to photosystem I through plastocyanin. More recently, it has been shown experimentally that cytochrome c_{6A} can indeed reduce plastocyanin, with a second-order rate constant comparable with that for the reduction of photosystem I by plastocyanin (Marcaida et al., submitted).

Structural analysis suggests that intramolecular electron transfer is possible. Structures determined to 1.2 and 1.4 Å resolution using X-ray crystallography for cytochrome c_{6A} with the haem oxidized (ferric form) or reduced (ferrous form) respectively have led to two important conclusions (Marcaida et al., submitted). The first is that oxidation or reduction of the haem does not lead to any significant change in the three-dimensional structure of the protein, and the LIP in particular. This argues against models in which the haem functions as a redox sensor, with changes in its redox state leading to conformational changes in the protein and transmission of a signal to some other molecule in the chloroplast [1]. The second conclusion is that the thiols in the LIP and the haem are sufficiently close for electron transfer from the thiols to the haem to be theoretically possible.

A final observation that underlies the model proposed here is that a number of proteins in the thylakoid lumen have the potential to form exposed disulphide bridges, and these may be required for optimal activity. For example, the immunophilin FKBP13 contains two disulphide bridges (at least one of which is exposed), the formation of which enhances the protein's peptidyl-prolyl isomerase activity [13]. The 33 kDa extrinsic PsbO protein of photosystem II (also known as the "manganese stabilizing protein" and the "oxygen-evolving enhancer protein") also has a disulphide bridge that has been shown to play an important structural role, at least in cyanobacteria [14–16]. The Rieske iron–sulphur protein contains a disulphide bridge, the equivalent of which in the cytochrome bc_1 complex is required for stability [17,18]. Thioredoxin affinity chromatography and other experiments also indicate the potential existence of disulphide bridges in a number of thylakoid lumen proteins, including additionally the thioredoxin-like protein Hcf164 and a 17.4 kDa protein of unknown function [16,19]. It has been suggested that, whereas reduction of disulphide bridges is required for maximal activity of a number of chloroplast stromal proteins, *formation* of disulphide bridges is generally required for maximal activity of lumen proteins [20].

3. The hypothesis

We suggest that the function of cytochrome c_{6A} is to catalyse the formation of disulphide bridges in proteins of the thylakoid lumen. The formation of a disulphide bridge in a target protein results in the reduction of the LIP disulphide by an exchange reaction. The resulting thiols in the LIP are then reoxidized, with the reducing equivalents passed to plastocyanin and thence to Photosystem I (Fig. 1). This reaction would be strongly favoured thermodynamically if we assume a midpoint potential for the thiol/disulphide group of cytochrome c_{6A} similar to that of thioredoxin, i.e. approximately −300 mV [21]. One possibility is that both electrons are transferred sequentially to the haem and then to plastocyanin, with the formation of a disulphide anion radical intermediate ($RSSR^{\cdot-}$). However, for simple dithiols in solution, the formation of the disulphide anion radical is energetically unfavourable (midpoint potential for the reaction

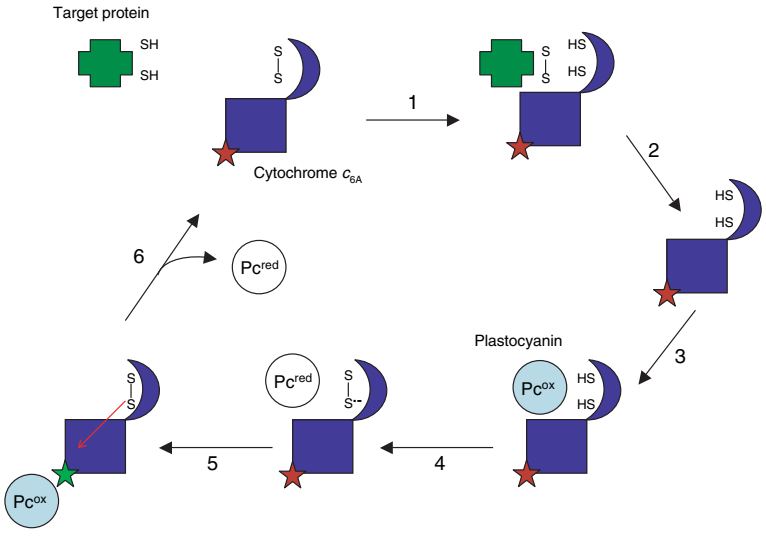

Fig. 1. Scheme for the function of cytochrome c_{6A}. In stage (1) a disulphide is formed in the target protein by exchange with cytochrome c_{6A}, leaving (2) cytochrome c_{6A} in the dithiol form. In stage (3) oxidized plastocyanin binds and removes a single electron in a slow reaction (4) generating a disulphide anion radical. This is followed by rapid electron transfer to the haem (5), which helps pull reaction (4) over and leads to resolution of the radical and reduction of the haem (green star). In stage 6, the reduced haem is reoxidized by plastocyanin.

$RSSR^{\cdot-} + e^- = 2\ RS^-$, where R is $HOCH_2CH_2$–, is +600 mV [22]). This implies that the restoration of the disulphide within cytochrome c_{6A} by sequential transfer of two electrons to the haem would be limited by the kinetics of the first transfer (even though the second transfer, from disulphide anion radical to haem, as well as the overall driving force, would be favourable). However, the first step could be speeded up by direct oxidation of one of the thiols of the LIP by plastocyanin, rather than by the haem of cytochrome c_{6A} (Fig. 1). In this case, the driving force would be ~250 mV higher than for reduction of the haem, and is predicted to give a rate 150 times faster. Therefore we propose that the first step is removal of one electron by direct interaction between plastocyanin and the LIP (this may also be facilitated by a ring of conserved aromatic residues around the base of the LIP). The second step would be rapid reduction of the haem, pulling the reaction over to completion. Once the second electron is on the haem, it would be stable and could be removed in due course by a second plastocyanin. The overall reaction is still the reoxidation of cytochrome c_{6A} by two molecules of plastocyanin. The presence of the haem minimizes the time for which the potentially dangerous radical exists.

A role for copper in the formation of disulphide bonds has been demonstrated elsewhere, for example in superoxide dismutase, although it is not clear whether the oxidation state of the copper changes [23]. If plastocyanin can oxidize the dithiol of cytochrome c_{6A} directly, it would not be unreasonable to suggest it could also oxidize the target protein directly, as shown in Fig. 2. This would account for the lack of a phenotype of a cytochrome c_{6A} mutant under normal conditions. However, as shown in Fig. 2, this reaction would form a radical intermediate on the target protein, requiring reaction with a second plastocyanin molecule. If plastocyanin concentrations were low, there would be a delay before a second plastocyanin molecule encountered the target protein, during which time the radical intermediate might follow side reactions resulting in the generation of reactive oxygen species, or damage to the target protein. Damage to FKBP13 would probably not be lethal, as reduction of FKBP13 to undetectable levels by RNAi is not lethal [24]. It seems more likely that lethality results from damage to PsbO or the Rieske iron–sulphur protein and consequent photoinhibition. Hence, the model explains why the cytochrome c_{6A} mutation is lethal in a background where plastocyanin levels have been depleted by RNAi [7]. Reduction in the level of plastocyanin is not lethal in the presence of cytochrome c_{6A}. Cytochrome c_{6A} removes both reducing equivalents from the target protein in a single reaction. Once the first reducing equivalent is removed from cytochrome c_{6A} by plastocyanin, the radical intermediate is immediately discharged by reduction of the haem, without having to wait for collision with a second plastocyanin molecule.

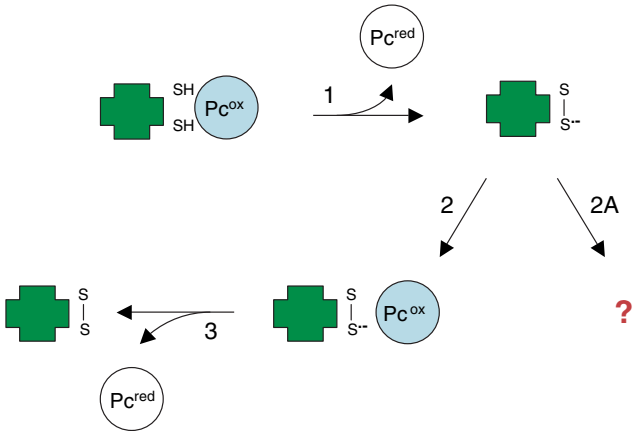

Fig. 2. Formation of disulphides in the absence of cytochrome c_{6A}. In stage (1) a single electron is removed by plastocyanin, generating an accessible reactive intermediate. This requires interaction with a second plastocyanin (2) for its resolution (3), but may enter damaging side-reactions (2A) if plastocyanin does not bind to the target protein in time.

Cytochrome c_{6A} can therefore be thought of as a 'funnel' for coupling the two-electron reaction of disulphide bond formation to the single-electron turnover of plastocyanin. Although under normal conditions, this 'funnel' is not essential, it becomes important when plastocyanin levels are depleted, and these are also the conditions under which the evolutionary precursor of cytochrome c_{6A}, i.e. cytochrome c_6, would have been induced. The presence of cytochrome c_{6A} may also be important when particularly high rates of biogenesis of the photosynthetic machinery are required, for example during rapid greening of young seedlings. It is possible, although not a requirement of our model, that synthesis of cytochrome c_{6A} is activated under such conditions. If the lethality in a background with reduced plastocyanin levels were, in effect, the result of photoinhibition, we might also expect a deficiency of cytochrome c_{6A} to have more effect at high light levels.

Another attractive feature of the model is that it provides a justification for the low redox potential of the haem of cytochrome c_{6A}. If cytochrome c_{6A} were reduced to its ferro-form by the cytochrome b_6f complex (which would happen with a conventional cytochrome c_6), its ability to act as a sink for the electrons of disulphide formation would be lost. The reduction in haem midpoint potential of cytochrome c_{6A} compared to the ancestral cytochrome c_6 therefore stops reduction of haem by the cytochrome b_6f complex.

4. Evolution of the system

Cytochrome c_{6A} has not yet been reported from cyanobacteria. It therefore seems likely that it evolved after the origin of chloroplasts, by duplication of the cytochrome c_6 gene, resulting in a situation similar to that seen in *Chlamydomonas*, which has the genes for both cytochrome c_6 and cytochrome c_{6A}. Selection then acted to improve the function of one copy for thiol oxidation. A short insertion resulted in the acquisition of a stretch of amino acids containing two appropriately placed cysteine residues, and the midpoint redox potential was lowered. It is not clear how cyanobacterial and non-green algal lineages that lack cytochrome c_{6A} generate disulphide bridges in the thylakoid lumen. It may be that disulphide bond formation is less important in those organisms, or that direct reaction with plastocyanin or cytochrome c_6 is sufficient to generate them.

5. Conclusion

We have proposed a novel hypothesis for the function of cytochrome c_{6A} in plants and green algae. Attractively, it explains

existing results on mutant phenotypes, and provides an explanation for the differences in cytochrome c_{6A} compared to conventional cytochrome c_6. It is consistent with structural and biochemical data, and makes a number of predictions that can be tested.

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